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Biochemical Studies of the Tracheobronchial Epithelium

by Marc J. Mass* and David G. Kaufman*

Tracheobronchial epithelium has been a focus of intense investigation in the field of chemical carcinogenesis. We have reviewed some biochemical investigations that have evolved through linkage with carcinogenesis research. These areas of investigation have included kinetics of carcinogen metabolism, identification of carcinogen metabolites, levels of carcinogen binding to DNA, and analysis of carcinogen–DNA adducts. Such studies appear to have provided a reasonable explanation for the susceptibilities of the respiratory tracts of rats and hamsters to carcinogenesis by benzo(a)pyrene. Coinciding with the attempts to understand the initiation of carcinogenesis in the respiratory tract has also been a major thrust aimed at effecting its prevention both in humans and in animal models for human bronchogenic carcinoma. These studies have concerned the effects of derivatives of vitamin A (retinoids) and their influence on normal cell biology and biochemistry of this tissue. Recent investigations have included the effects of retinoid deficiency on the synthesis of RNA and the identification of RNA species associated with this biological state, and also have included the effects of retinoids on the synthesis of mucus-related glycoproteins. Tracheal organ cultures from retinoid-deficient hamsters have been used successfully to indicate the potency of synthetic retinoids by monitoring the reversal of squamous metaplasia. Techniques applied to this tissue have also served to elucidate features of the metabolism of retinoic acid using high pressure liquid chromatography. In brief, formidable strides have been made in biochemistry specific to this important target tissue, despite the inability to acquire tracheobronchial epithelium in large quantities.

Introduction

The tracheobronchial epithelium is a primary interface with the environment, its constituents and its contaminants. It is, then, not surprising that this tissue gives rise to more fatal malignancies than any other tissue in the human body. Cancers of the respiratory tract cause 14% of cancer deaths in women and 34% in men (*1*). Because the vast majority of lung cancers are, in reality, cancers of the cells lining the large airway passages (*2*), a concerted effort has been directed at achieving experimental models of human lung cancers. The intratracheal instillation technique of Saffiotti et al. (*3*), the heterotopic tracheal graft model of Nettesheim et al. (*4*) and the localized instillation technique of Schreiber et al. (*5*) all produce cancers with histologic similarities to human bronchogenic epidermoid carcinoma. This review will consider the development of experimental methods and the pursuit of two lines of investigation which have evolved from these experimental models of lung cancer. The first of these investigative issues concerns the activation of carcinogens in this target tissue, and the second relates to the action of a class of agents, retinoids, that may have promising cancer chemopreventive properties in this tissue.

We review here findings of some current investigations in which epithelial cells of the tracheobronchial airways have been used as a source for various biochemical analyses. These investigations have included carcinogen metabolism, binding and adduct analysis, glycoprotein biosynthesis, RNA synthesis, bioassay of retinoids and retinoic acid metabolism.

Biochemical studies in the tracheobronchial epithelium appear, upon first consideration, to be a rather limited proposition because of the small quantity of tissue that is available. However, the studies mentioned below attest to the fact that it is possible to perform some sophisticated analyses in this tissue.

Methods for Biochemical Study of Tracheobronchial Epithelium

The tracheobronchial epithelium is far more difficult to use for biochemical studies than solid organs. The cells comprising this tissue are those which line the major pulmonary airways as well as connective tissue, smooth muscle, and cartilage. Those biochemical characteristics specific to the epithelial cells can be appreciated if these cells can be separated from their supporting elements. Yet, such a procedure yields small

*Department of Pathology, University of North Carolina School of Medicine, Chapel Hill, NC 27514.

quantities of cells, using many animals, a large expenditure of time, and considerable cost. Nonetheless, biochemical methods have been developed for studies with this tissue (Table 1).

Initially, studies of biochemical processes utilized the intact trachea in large laboratory animals. Wardell et al. (*6*) studied tracheal mucus production in dogs by collecting secretions from grafted tracheas. Excised segments of the trachea were implanted subcutaneously to form closed pouches, or sewn such that the lumen of the trachea communicated with the outside through a tracheocutaneous fistula. Secretions were collected from the heterotopically placed tracheas and analyzed. Recently, studies of glycoprotein biosynthesis by Clark and Marchok (*7*) utilized a modification of this graft technique but with rat tracheas. These investigators were able to collect milligrams of tracheal mucus for subsequent analyses.

Table 1. Examples of methods used for tracheobronchial biochemistry.

Procedure	Species	References
Isolation of epithelial cells		
Mechanical isolation	Hamster	(*8,13*)
Enzymatic dissociation	Hamster	(*11,29*)
	Rat, mouse, rabbit	(*32*)
Isolation of cell components		
Nuclei	Hamster	(*8*)
Microsomes	Hamster	(*13*)
Cell homogenates	Hamster	(*13,14,20*)
Macromolecule isolation		
DNA	Hamster	(*24*)
RNA	Hamster	(*73,88*)
Glycoproteins	Hamster	(*11,84,89,90*)
	Rat	(*7,10,76,77*)
	Dog	(*6*)
	Rabbit	(*32,91*)
Glycerolipids	Rat	(*92*)
Carcinogen metabolism		
Carcinogen–DNA binding		
BP	Hamster	(*13,14,24,28*)
BP	Rat	(*14,28*)
BP	Bovine	(*28,93*)
BP	Human	(*15,28,59*)
BP	Mouse	(*28*)
DMBA	Human	(*20*)
Nitrosamines	Human	(*54*)
TLC analysis		
BA, 7-MeBA, BP	Human, rat, hamster	(*36*)
BP	Human, rat, hamster	(*17–19*)
DMBA	Rat	(*44*)
HPLC analysis		
BP	Human	(*20,28*)
BP	Hamster	(*13,14,28*)
BP	Rat	(*14,17,28*)
BP	Mouse	(*28*)
Carcinogen–nucleoside adduct analysis		
BP	Bovine	(*93*)
BP	Human	(*59*)
BP	Hamster, rat, mouse	(*28*)
Aflatoxin B_1	Human	(*55*)
DNA repair		
Unscheduled synthesis	Rat	(*94*)
BP–DNA adduct removal	Hamster	(*33*)
Retinoic acid metabolism	Hamster	(*34,35,37,80*)

To study subcellular and macromolecular elements specifically originating from the epithelial cells, it was necessary to have a technique to isolate these cells from the rest of the trachea. Smith et al. (*8*) were able to recover a tracheal cell population by scraping the epithelium from the tracheal surface of Syrian hamsters. Nuclei isolated from the tracheal cells were morphologically intact and competent at the synthesis of RNA. Based on earlier observations that tracheal organ cultures could be maintained *in vitro* with reasonable morphologic preservation, Kaufman et al. (*9*) showed that excised hamster tracheas incorporated radioactively labeled macromolecular precursors from culture medium during relatively brief periods of incubation *in vitro*. Precursors of protein, RNA and DNA were incorporated into these macromolecules within tracheal epithelial cells and were analyzed in cells scraped from the tracheas after the incubation period. Autoradiographic studies performed simultaneously with biochemical studies showed that thymidine was largely incorporated into basal cells, uridine was most actively incorporated into the nuclei of basal cells and ciliated cells, whereas leucine was most actively incorporated into mucous cells. These initial studies also showed that macromolecules could be sufficiently labeled in this manner to permit more sophisticated biochemical studies.

Subsequently, Bonanni et al. (*10,11*) showed that when relatively stable macromolecular products such as glycopeptides were the object for study, such products could be isolated from tracheal epithelial cells that were enzymatically dissociated from the underlying supportive structures by incubation with hyaluronidase. Even smaller quantities of tissue than those used above apparently have been sufficient for studies of gel-electrophoretic analyses of LDH isozymes in the bronchi of rats exposed intratracheally to benzo(a)pyrene (BP). In these studies, bronchial mucosa was isolated from rats by smearing the epithelium onto a slide, freeze-drying the slide, and scraping off the epithelial cell residue (*12*).

Thorough studies of carcinogen metabolism in tracheobronchial epithelium also became feasible as the result of organ culture of this tissue. Prolonged and reproducible exposures of the epithelium directly to carcinogens were possible, and since no other parts of the respiratory tract were present, the specific features of metabolism in this tissue could be evaluated. Studies of carcinogen metabolism have progressed further through the development of methods for preparation of epithelial cell homogenates from this tissue and isolation of microsomes. Typical kinetic evaluations of BP monooxygenase have been feasible with microsomal

fractions from rat and hamster tracheas (*13,14*), and the activity of the monooxygenase was detectable in homogenates of cells scraped from human bronchus (*15,16*). Specific products of the biotransformation of radiolabeled carcinogens have been separated and identified by thin layer and high-pressure liquid chromatography (*13,14,17–23*). The binding of the carcinogen BP to DNA was quantitated in cells isolated from hamster tracheas by the scraping technique (*24*). DNA was purified from tracheal cells by phenol extraction, solvent extraction of unreacted BP, enzymatic removal of protein and RNA, followed by banding at equilibrium in centrifugally generated CsCl gradients. These techniques were applied to the isolation of DNA from human bronchial tissues (*15,20,25–27*). Though the quantities of DNA isolated are often on the order of micrograms, this has proven sufficient to allow BP–DNA adduct analyses in a variety of species including hamsters, rats, mice, bovines and humans (*28*).

With the advent of cell culture techniques for respiratory epithelial cells (*29–32*), defined populations of cells from this tissue have been evaluated for their metabolic characteristics with respect to BP (*18*). Recently, Eastman et al. (*33*) utilized hamster tracheal cells in monolayer culture to perform the first reported studies of repair of carcinogen–DNA adducts in respiratory epithelial cells.

Sporn and colleagues (*34–40*) have been studying the mechanism of action of retinoids in the respiratory epithelium. Organ cultures of the tracheobronchial epithelium from hamsters were found suitable as exquisitely sensitive bioindicators of the potency of retinoids. Studies on the metabolism of retinoic acid and its active intermediates (*34,35*) have proceeded somewhat like studies of carcinogen metabolism with metabolites of retinoic acid extracted from culture medium by organic solvents and analyzed by HPLC.

The preceding discussion of biochemical methods employed for studies of tracheobronchial epithelium indicates the progress that has been made in this area of biochemistry. The following two sections illlustrate more thoroughly two areas of research on the biochemistry of the tracheobronchial tract that have shown notable progress.

Carcinogen Metabolism in Tracheobronchial Epithelium

Biological Evidence of Metabolism of Polynuclear Hydrocarbons in Respiratory Epithelium of Rodents

The lung consists of at least 40 cell types (*41*). A proportionately large share of these cells provides structural support for lung architecture and lines the capillary network which interfaces the air spaces to the blood supply. It is the cells which line the trachea and bronchi which are at manifestly greater risk for the development of lung cancer, but the cells of the tracheobronchial epithelium comprise a minority of the total cell population within the lung. Studies of carcinogen activation in whole lung homogenates or subcellular fractions thereof, do not directly reveal the characteristics of polynuclear hydrocarbons (PNH) metabolism in the cells most vulnerable to carcinogens, the cells lining the tracheobronchial airways.

BP, a ubiquitous carcinogen and constituent of cigarette smoke, serves as a most well characterized marker for the presence of PNH in the environment. Until recently there has been little information acquired concerning BP metabolism in respiratory tract tissues where this agent may exert a carcinogenic effect *in vivo*.

Wattenberg and Leong (*42*) in 1962 attempted to identify and localize sites of PNH metabolism in frozen sections of rodent tissues using a histofluorescent assay for "pyridine nucleotide dependent polycyclic hydrocarbon metabolizing systems." They were unable to demonstrate that respiratory epithelium metabolized BP since these cells lacked fluorescent characteristics of BP metabolites, but metabolic capacity was observed in alveolar walls. However, studies of respiratory carcinogenesis by Saffiotti et al. (*3*) in which BP–ferric oxide was administered intratracheally to hamsters implied that the trachea and bronchi could metabolize or, at least, respond to this carcinogen by virtue of the development of tracheobronchial cancers following such carcinogen treatments. Theoretically, enzymatic activation could have occurred elsewhere in the respiratory tract and activated metabolites could have entered tracheal or bronchial cells by diffusion. Alternatively, pulmonary macrophages which scavenged the instilled carcinogen-laden particulate material could have released active metabolites (*43,44*). More direct evidence of the capacity for carcinogen metabolism in tracheal epithelium was provided by Dirksen and Crocker (*45*) and by Palekar et al. (*46*). These investigators observed that tracheal organ cultures of rat or hamster maintained in medium containing 7,12-dimethylbenz(a)anthracene (DMBA), benz(a)anthracene (BA), BP, and 3-methylcholanthrene (MCA) exhibited morphologic changes of hyperplasia, metaplasia and other general cytotoxic reactions. This observation narrowed the likely site of activation since other elements of the respiratory tract were absent. The alterations in this tissue, therefore, were most probably related to the conversion of the PNH to toxic metabolites directly in tracheal cells. These results have been reproduced and extended in more recent years by Lane and Miller (*47,48*), and by Mossman and Craighead (*49*) with BP and MCA, respectively.

More biologically integrated studies of carcinogen activation in respiratory epithelium began to explore animal models for human lung cancer. Biochemical and anatomic factors which contributed to susceptibility to carcinogen-induced neoplasia in animals were character-

ized and considered with respect to their relevence to humans. Studies in hamsters with the BP–ferric oxide model of Saffiotti et al. (*3*) demonstrated that the trachea was an ideal target tissue for biochemical investigations. Subsequent studies have used human respiratory tissues in explant culture.

Carcinogen Metabolism and DNA Binding in Rodent Respiratory Tissues

Kaufman et al. (*24*) gave the first biochemical evidence of the capacity of tracheal epithelium to metabolize BP. Excised hamster tracheas were incubated in tissue culture medium containing [^{3}H]BP, and epithelial cells were subsequently obtained by scraping the mucosal surface of tracheas and the DNA from these cells was isolated and purified in CsCl gradients. The DNA had radioactivity associated with it which resisted repeated ether extraction and was therefore attributed to covalently bound [^{3}H]BP. Prior treatment of hamsters with intratracheal BP–ferric oxide enhanced the binding of [^{3}H]BP to DNA in tracheal epithelial cells. This observation demonstrated substrate inducible metabolism, a feature of cytochrome P-450-mediated reactions. That this enzyme complex was involved in the carcinogen binding was confirmed by the observation that 7, 8-benzoflavone (7,8-BF) inhibited the binding of [^{3}H]BP to DNA in tracheas from BP–ferric oxide-pretreated animals but not in tracheas from control animals. This flavone inhibits the metabolism of PNH catalyzed by liver microsomes obtained from rats pretreated with an intraperitoneal injection of MCA. Autoradiographic analysis (*15,27*) demonstrated morphologically that [^{3}H]BP was bound to cytoplasmic and nuclear constituents primarily in the epithelial cells. The extent of binding of [^{3}H]BP in these autoradiographic studies was reduced by co-incubation with 7,8-BF; this finding was consistent with the biochemical studies.

Harris et al. (*20*) used a fluorometric assay (*50*) to measure aryl hydrocarbon hydroxylase (AHH) activity in homogenates of cells scraped from bronchial organ cultures. The radiometric assay of Hayakawa and Udenfriend (*51*) was used to measure metabolites released into the organ culture medium from explants of bronchial epithelium (*20,43*). The fluorometric assay sensitively measures phenolic products of BP metabolism, and the assay of Hayakawa and Udenfriend (*51*) measures the production of tritiated water resulting from oxygenated ^{3}H-hydrocarbon metabolites which have undergone NIH shift concomitantly with tritium loss upon formation. These assays offered a measure of the formation of BP phenols and quinones by tracheal tissue with the greatest sensitivity then available. These methods, however, did not permit the various products to be distinguished; some products, for example, dihydrodiols, retain tritium upon formation, and their presence was undetected.

Pal et al. (*22*) were the first to separate the products of metabolism of PNH to further characterize these metabolic processes in tracheobronchial epithelium. They showed that thin-layer chromatography (TLC) could be used to separate and identify phenol and dihydrodiol metabolites of BA, 7-MeBA, and BP produced by organ cultures of human bronchus and by rat and hamster trachea. Cohen et al. (*19*) used organ cultures of tracheas, bronchi and peripheral lung from rats, hamsters and humans as well as isolated perfused rat and hamster lung in studies of BP metabolism. BP metabolites, separated by TLC showed qualitative similarities in metabolite patterns between tissues and species. Qualitative differences were found between metabolism in hamster lung fragments and in tracheas and bronchi; this generally and perhaps fortuitously corresponded to the relative susceptibility of these tissues to tumorigenesis by BP in these tissues. Although a low level of BP metabolism was detected in lung tissue, this was of uncertain significance because early signs of necrosis (darkened nuclei) were seen in histologic sections of the lung fragments, and this would account for the low level of metabolism. In another report, Cohen et al. (*17*) identified an ethyl acetate-soluble conjugate between sulfate and monohydroxy BP that was a major metabolite in organ cultures of respiratory tissues. Despite the identification of products of metabolism, these observations offered only a limited view of carcinogen metabolism in respiratory tract tissues for two reasons. First, TLC methods offered qualitative identification of products but quantitative measurements were not readily made. Second, since the underlying microsomal metabolism had not been evaluated, the pattern of metabolites, particularly those conjugated forms released into the organ culture medium, could not be related to the primary products of BP metabolism by microsomes.

The assay for BP metabolism developed by DePierre et al. (*52*) quantitated all of the products formed with great improvement in sensitivity and precision. This offered the possibility for direct measurement of BP metabolism by microsomes from tracheobronchial epithelium. Microsomes were isolated by Mass and Kaufman (*13,14*) from epithelial cells scraped from rat or hamster tracheas; the yield of microsomal protein was about 5 to 10 μg per trachea. By using this more sensitive radiometric assay, metabolism of BP by microsomes from hamster tracheas was detectable; furthermore, Lineweaver-Burk plots were prepared and K_m and V_{max} values were estimated. Preincubation of tracheas with BP prior to isolation of microsomes doubled the V_{max} of the monooxygenase; nonetheless the activity of tracheal microsomes was 50-fold lower than the activity of microsomes from the livers of rats pretreated with IP injections of MCA. K_m values for microsomes from hamsters fell between 1 and 2 μM, suggesting that the monooxygenase has a high affinity for BP. Similar studies with microsomes from rat tracheas never detected metabolism, presumably because the activity of benzopyrene monooxygenase in rat

tracheal microsomes is below the limit of detection of this assay. The activity was detected in 10,000*g* supernatants of rat tracheal cell homogenates, but only if the trancheas were preincubated with BP prior to cellular isolation. The metabolic activity of supernatants from rat and hamster tracheal cell homogenates are compared in a Lineweaver-Burk plot. (Fig. 1) The BP-induced component of the mixed-function oxidase in rat tracheal cells was half that of hamsters; however, the K_m values, spanning the 1 to 2 μM range, were essentially equivalent. Titration of 7,8-BF effects on benzopyrene monoxygenase from 10,000*g* supernatants of hamster tracheas (Fig. 2) showed results like those previously seen for DNA binding (*24*). BP pretreatment increased the sensitivity of benzopyrene monooxygenase to 7,8-BF.

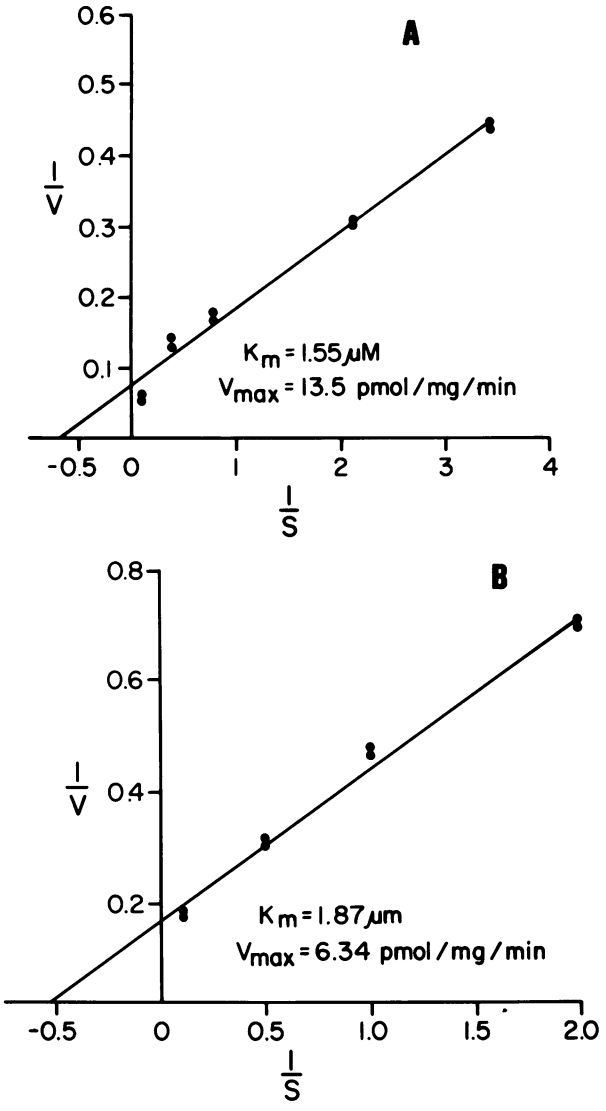

FIGURE 1. Lineweaver-Burk plot for benzopyrene monooxygenase activity in supernatants of tracheal epithelial cell homogenates centrifuged at 10,000*g*: (*A*) BP-pretreated hamster tracheas: (*B*) BP-pretreated rat tracheas. Ordinate expressed as min/pmole; abscissa, inverse substrate concentration expressed as 1/μM.

The adoption of HPLC analysis of metabolites produced by hamster trancheal microsomes showed that about 50% of the metabolites produced by hamster tracheal microsomes cochromatograph with quinone derivatives of BP (Fig. 3). BP-phenols (3-OH and 9-OH) comprised the second major class of metabolites and these were well separated from dihydrodiols (diols) of BP, the next most prevalent class of oxygenated products. Metabolites produced by microsomes isolated from BP-induced rat tracheal organ cultures had HPLC profiles that differed from those produced by hamster tracheal microsomes (Fig. 4). Rat tracheal microsomes produced almost exclusively 3-OH BP with only a minute peak cochromatographing with the 9,10-diol of BP.

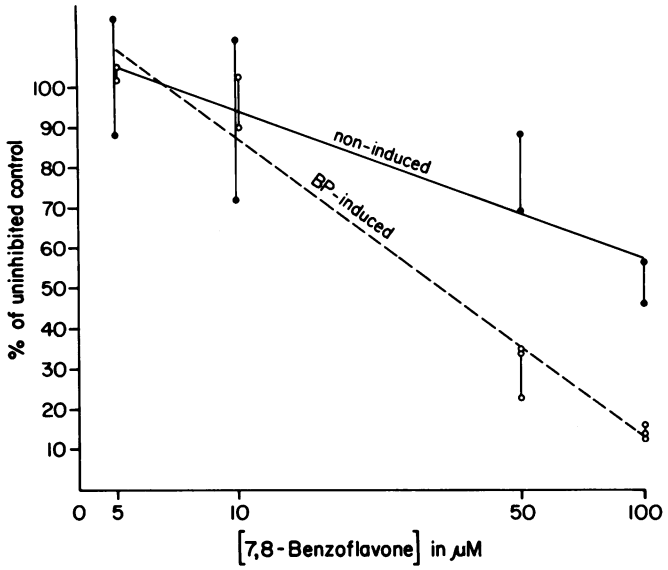

FIGURE 2. Effect of 7,8-BF on benzopyrene monooxygenase activity in supernatants from 10,000*g* centrifugation of hamster tracheal epithelial cell homogenates. Incubation time was 5 min at 37°C; concentration of [^{3}H]BP was 5 μM.

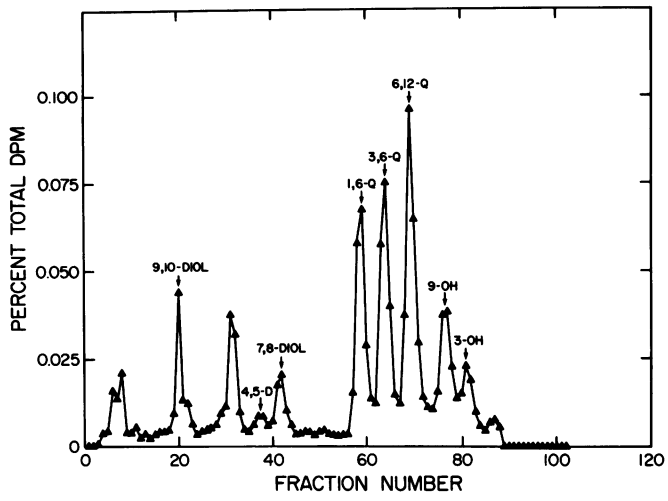

FIGURE 3. Metabolites produced by microsomes from BP-pretreated hamster tracheas after 30 min incubation with [^{3}H]BP and cofactors at 37°C. Separation by reverse-phase HPLC.

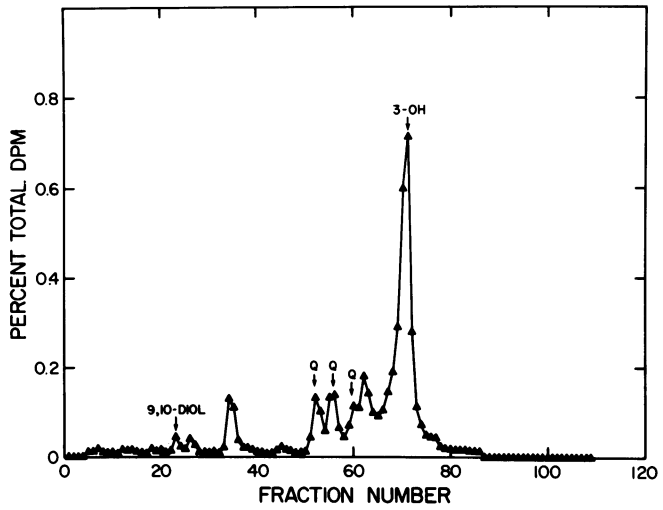

FIGURE 4. Reverse-phase HPLC separation of metabolites produced by rat tracheal microsomes isolated from BP-pretreated tracheas. Microsomes were incubated for 30 min with [^{3}H]BP and cofactors at 37°C.

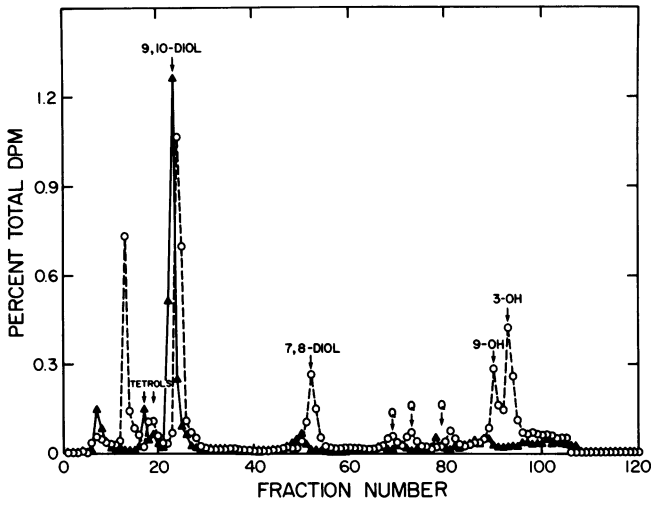

FIGURE 5. Hamster tracheas were incubated with 1 μM [^{3}H]BP for 24 hr and variously treated organ culture medium: (▲) extracted with ethyl acetate/acetone and organic solvent soluble metabolites separated by reverse-phase HPLC; (○) exposed to 11 units of aryl sulfatase and 1000 units of β-glucuronidase at pH 5 for 2 hr prior to extraction with ethyl acetate/acetone and separation by HPLC.

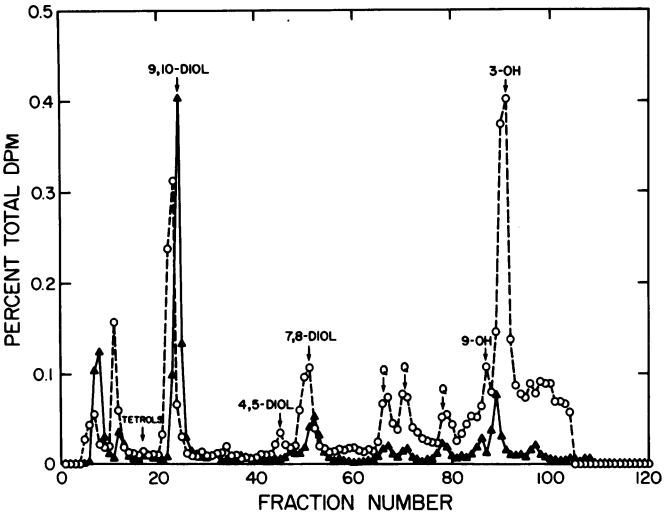

FIGURE 6. Rat tracheas were exposed to 1 μM [^{3}H]BP in organ culture medium for 24 hr: (▲) radioactivity from ethyl acetate/acetone extract of medium separated by reverse-phase HPLC; (○) organ culture medium exposed to 11 and 1000 units of β-glucuronidase and aryl sulfatase, respectively, and metabolites extracted with ethyl acetate/acetone, and separated by reverse-phase HPLC.

To determine whether the observed differences between rat and hamster in the activity microsomes and the distribution of products formed were reflected by the metabolism in intact tracheal cells, further studies of metabolism were done with tracheas in organ culture. After rat and hamster tracheas had been incubated with [^{3}H]BP, a portion of the medium was extracted with ethyl acetate/acetone and the remainder was treated with β-glucuronidase and aryl sulfatase prior to extraction. By comparing the metabolites extracted in these two ways, it was possible to determine both the quantities and proportions of water-soluble metabolites released into the ethyl acetate/acetone-soluble phase by the deconjugating enzymes. About 35% of the metabolites released into the medium by organ cultures were ethyl acetate/acetone-soluble, and the remainder were water-soluble. Deconjugation by aryl sulfatase and β-glucuronidase released an additional 30% of the total products; nearly 40% of the metabolites were still water-soluble after the enzyme treatment. The metabolites which were conjugated were mostly BP-phenols, BP-quinones, 7,8-diol, and highly polar derivates, some of which were polyhydroxylated (Fig. 5). The quantity of 9,10-diol which was ethyl acetate/ acetone-extractable did not change after the enzyme treatment. Rat tracheal organ cultures produced less ethyl acetate/acetone- and water-soluble metabolites per millgram of tracheal tissue than did hamster tracheal organ cultures (a total of 3.7 pmole/mg/24 hr and 7.8 pmole/mg/24 hr for rat and hamster, respectively). The major ethyl acetate/acetone-extractable metabolite was the 9,10-diol (Fig. 6). BP-phenols and a peak which contains a monohydroxy BP sulfate conjugate (BP–SO_4) comprised the next most abundant group of metabolites. Treatment of the medium with aryl sulfatase and β-glucuronidase released an additional 35%, but 43% of the radioactivity was still water-soluble. Enzyme treatment of medium from rat tracheal organ cultures released 3-OH BP as the major microsomal metabolite with lesser quantities of BP-quinones released. Virtually no BP-tetrols were present in the culture media (< 1.5 pmole), whereas hamster tracheas produced 15 pmole. The ratio of ethyl acetate/ acetone-soluble metabolites released from medium by aryl sulfatase and β-glu-

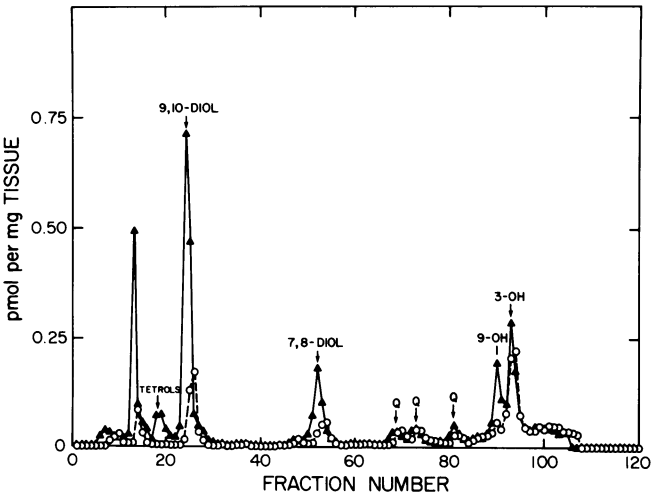

FIGURE 7. Comparison of metabolites produced by (▲) hamster tracheas or (○) rat tracheas after exposure of organ culture fluid to β-glucuronidase and aryl sulfatase. The results have been normalized for the weight of tissue utilized in the incubation.

Table 2. Comparison of metabolites produced by rat and hamster tracheal organ cultures.

Metabolite	Metabolite produced, pmole/mg tissue/24 hr		Ratio (hamster/rat)
	Hamster	Rat	
Pre-tetrols	0.86	0.18	4.78
Tetrols	0.22	0.01	22.0
9,10-Diol	1.41	0.30	4.70
4,5-Diol	0.05	0.04	1.26
7,8-Diol	0.46	0.15	3.07
Quinones	0.45	0.30	1.50
9-OH	0.48	0.11	4.36
3-OH	0.61	0.56	1.09

curonidase varied from 1.09 to 22.0 for hamster versus rat tracheas (Table 2). The greatest discrepancy in ratios is that of BP-tetrol formation; hamster tracheas produced 22-fold more BP-tetrols than did rat tracheas. This difference in products is further amplified in Figure 7.

In light of differences in metabolism noted for rat and hamster tracheas, studies of binding of BP to DNA of tracheas in culture were performed to further compare these two species. DNA was isolated from epithelial cells and banded on isopyknic CsCl gradients. The mean binding level for rat tracheas was 1.55×10^{-4} pmole bound/μg DNA. A separate experiment to determine the effect of a pretreatment with BP on binding levels revealed a binding level of 1.23×10^{-4} pmole/μg DNA in tracheas pretreated with BP whereas control tracheas had a level of 0.91×10^{-4} pmole/μg DNA. In hamster tracheas BP-pretreatment resulted in a greater than 2-fold increase in binding (16.9×10^{-4} versus 42.6×10^{-4} pmole/μg DNA) which is in substantial agreement with the earlier study by Kaufman et al. (*24*). The mean binding level for hamster tracheas was 26.7×10^{-4} pmole/μg DNA. The 17-fold greater binding to DNA in hamster tracheas than rat tracheas was comparable to the ratio of BP-tetrol formation in these two species and may explain the species specificity for BP-induced carcinogenesis in the tracheas of hamsters (*14,53*).

Carcinogen Metabolism and Binding in Human Respiratory Tissues

A series of recent experiments have extended the study of metabolism and the binding of the activated carcinogen to cellular DNA to human bronchial tissue. These studies were of obvious importance because the ultimate goal of previous studies, realized in these investigations, is knowledge of the metabolism of carcinogens in the critical human target tissue. Harris et al. (*27*) demonstrated that specimens of human bronchus could catalyze the binding of PNH to DNA of epithelial cells in explant culture of human bronchus. The specific activity of binding of 4 PNH [DMBA, BP, MCA and dibenzanthracene (DBA)] to bronchial cell DNA in two to four unique specimens was assessed and had a mean of 40 pmole/10 mg DNA; however, the specimens bound more DMBA and BP than MCA or DBA (*27*). In a study of the levels of binding of BP and DMBA assessed in 28 specimens of human bronchus, the mean level for DMBA–DNA binding was 118 pmole/10 mg DNA and that of BP–DNA binding was 38 pmole/10 mg DNA, indicating that DMBA was bound to a significantly greater extent. The levels of binding of BP to bronchial DNA were studied more intensively in 37 specimens from patients with or without lung cancer (*25*). The specimens were obtained either after immediate autopsy or during surgery to ensure the viability of the tissues. The tissues were placed in culture for 7 days prior to the addition of [^{3}H]BP to the culture medium to reduce the contribution by any exogenous or endogenous factors which could influence the levels of BP–DNA binding (i.e., specimens from smokers, or those taking medications). All tissues were resected from normal segments of the bronchus when specimens from patients with lung cancer were used. The levels of binding of BP to DNA in these specimens of human bronchus varied by 75-fold. In these initial studies there was no obvious relationship between levels of binding in specimens from patients with or without lung cancer. A more recent report (*26*) using 79 specimens showed a 150-fold range of variation in BP–DNA binding. Interestingly, when specimens from lung cancer patients were segregated according to histologic type of the tumor, a difference was noted. Binding levels were higher than controls in specimens from patients with epidermoid carcinoma, epidermoid combined with adenocarcinoma, and in mucous differentiated cancers with a nonglandular pattern. The mean binding level in specimens from patients with glandular, mucous differentiated cancers was not significantly different from controls. The metabolism of both cyclic and acyclic *N*-nitrosamines was assessed in human bronchial organ cul-

tures and these compounds were found to be metabolized to products which bound to cellular DNA (*54*). The capacity of human bronchus to activate aflatoxin B_1 to derivatives which bind to DNA also was determined; the values obtained were somewhat lower than with BP (*55*).

The range of variation in levels of carcinogen binding to human bronchus was similar to those observed with other human tissues. There was a 99-fold variation in the binding of BP to DNA in cultured human esophagus (*56*), 100-fold in colon (*57*) and 70-fold in human endometrium (*58*). The absolute values for the binding of BP to DNA in human tissues were highest in human bronchus and esophagus, lower in endometrium, whereas human colon bound approximately 1/10 the quantity of BP to DNA than did bronchus. The mean binding level for human bronchus (25 pmole/10 mg DNA) was close to that seen for tracheas of Syrian golden hamsters in culture (*14*). The factors which govern the levels and variation in BP–DNA binding levels in human bronchus have not been determined. The levels of binding were induced by BA pretreatment and by BP itself, and the binding was time- and temperature-dependent (*15*). The variation was apparently not related to the formation of different BP–DNA adducts, since the major adduct in bronchus, esophagus and colon is an adduct of (±) 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)-pyrene with guanine (*28,56,57,59*). A likely determinant of the levels of binding of BP to DNA is variations in levels and species of cytochrome P-450. It has been observed that the total metabolic capacity for BP in microsomes from human lungs vary (*60*), and Genta (unpublished observations) (Fig. 8) has noted that 7,8-BF inhibits the binding of BP to DNA by 40% to 80% in bronchial specimens from nine individuals. Compounds other than 7,8-BF (*15*), including butylated hydroxytoluene (BHT) and nicotine, have been tested for their effect on BP–DNA binding in human bronchial organ cultures. The antioxidant BHT is a potent inhibitor of binding and reduced the level of BP–DNA binding by 80% at a concentration of 2 μM. Nicotine, although effective as an inhibitor of AHH in lung homogenates, did not alter the level of binding in organ cultures of human bronchus (*15*).

The metabolism of BP has been characterized by HPLC in organ cultures of human bronchus. As with rodent respiratory tissues, human bronchus produced organic solvent-extractable and water-soluble metabolites of BP (*20,28*). The ethyl acetate/acetone-soluble components consisted of the 9,10-diol of BP and the more polar triols and tetrols. Water-soluble metabolites consisted of sulfate, glucuronide, and glutathione conjugates of BP metabolites including BP-tetrols, the 9,10-diol, 4,5-diol, 7,8-diol, BP-quinones and BP-phenols (*28*). Approximately half of the BP metabolites were water-soluble, and half can be extracted with ethyl acetate/acetone. When specimens from patients with or without lung cancer were analyzed with respect to BP-metabolite profiles, they were indistinguishable from those from controls (*20*).

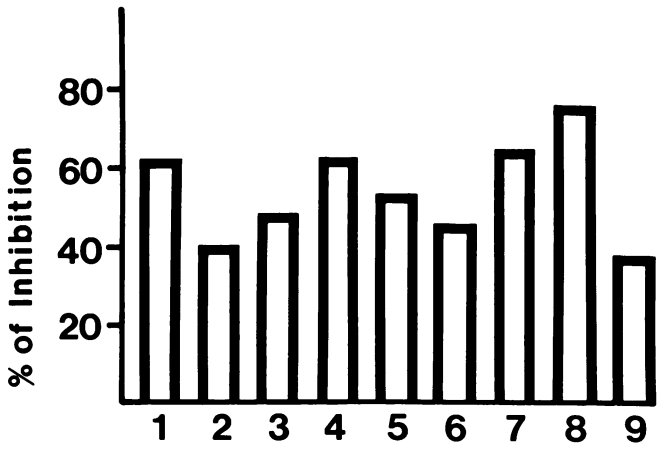

FIGURE 8. Inhibition by 7,8-BF of binding [^{3}H]BP to DNA in organ cultures of human bronchial epithelium from nine separate specimens. Ordinate, ratio of specific activity of BP binding to epithelial cell DNA in cultures with 7,8-BF added, to specific activity determined in control cultures × 100; abscissa, case identification number. Concentration of 7,8-BF and [^{3}H]BP were 2 μM; incubation period was 24 hr. Data of V. M. Genta (unpublished observations).

Effects of Retinoids on Tracheobronchial Epithelium

A second area concerned with the biology and biochemistry of tracheobronchial epithelium has been the subject of extensive study and progress in recent years. This is the various effects of retinoids on this tissue. The term "retinoids" has been used to describe the natural forms of vitamin A and the numerous functional and structural analogs of vitamin A that have been synthesized. Studies of retinoids have concerned biochemical and biological studies of retinoids, and investigations concerning their metabolism and mechanisms of action.

Relationship of Retinoids to Lung Cancer

The interest in the effects of retinoids on tracheobronchial epithelium stems from the observations of Saffiotti et al. (*61*) that administration of large doses of vitamin A to hamsters previously treated intratracheally with BP–ferric oxide caused a marked reduction in the number of respiratory tract tumors developing in this animals. Concurrent studies with natural and subsequent studies with natural and artificial retinoids have shown administration of high doses of retinoids to exert an inhibitory or preventive effect on carcinogenesis in several other organs (*38*). The subsequent record of studies of the effects of retinoid treatment on respiratory carcinogenesis has been less consistent. Retinoids

have been shown to inhibit respiratory carcinogeneis (*61–64*) or to have no effect (*65–67*) or even to potentiate respiratory carcinogenesis (*68*). The general interest in retinoids generated by these studies led to consideration of the effects of the vitamin A-deficient state with regard to carcinogenesis. Genta et al. (*69*) explored the effects of vitamin A deficiency on the binding of BP to DNA in tracheas from vitamin A-deficient and normal hamsters. Their observation, that vitamin A deficiency enhanced the binding of BP to DNA, led to further consideration of the effects of the vitamin A-deficient state on carcinogenesis in this tissue. Nettesheim et al. (*41,62*) evaluated this issue in rats maintained in marginal, near-deficient vitamin A status. In comparison to rats provided a roughly normal vitamin A intake, after intratracheal instillations of MCA, the rats nearly deficient in vitamin A developed a greatly increased incidence of squamous nodules and of squamous carcinomas of the peripheral lung. This observation has been supported by three epidemiologic studies which suggest a relationship between vitamin A status and lung cancer risk. Bjelke (*70*) conducted a prospective study in which vitamin A intake levels were estimated from dietary history surveys in a large Norwegian population and found that lung cancer incidence was inversely correlated with vitamin A intake. Mettlin et al. (*71*) estimated vitamin A levels from vegetable consumption surveys in a retrospective study on a separate population. Again, a low estimated vitamin A intake was associated with a higher relative risk for lung cancers among subjects who smoked cigarettes. In a recent prospective study (*72*) serum retinol levels were determined in a large geographically defined population. Low serum retinol levels were found among subjects who subsequently developed an increased incidence of cancers including lung cancer. This correlation was also consistent for the development of epithelial cancers of various cell types in several tissues with a high incidence of cancer.

RNA Metabolism and Inhibitors of RNA Synthesis

Efforts have been made to determine the mechanism of action of vitamin A in tracheal epithelium and to elucidate the relationship between vitamin A and carcinogenesis. On the macromolecular level, it was hypothesized (*9,24*) that the morphologic manifestations of vitamin A deficiency might be detectable as changes in the types and classes of proteins synthesized and that this might be distinguished in the quantity and species of their RNA precursors. To test this, tracheas were excised from vitamin A-deficient or normal hamsters and were incubated in organ culture medium containing [^{3}H]uridine. Epithelial cells were scraped from tracheas by the method of Smith et al. (*8*), whole cell RNA and DNA was isolated by phenol extraction, and nucleic acids were applied to agarose–acrylamide gels. In comparison to RNA species from normal tracheas, the profile of RNA resulting from incorporated [^{3}H]uridine in gels from tracheal epithelial cells of vitamin A-deficient hamsters consistently had reduced levels of certain high molecular weight RNA species (Fig. 9). This difference in gel profiles could be reversed by treatment of vitamin A-deficient animals with retinyl acetate, restoring the proportion of high molecular weight RNA species.

To determine if vitamin A deficiency affected the

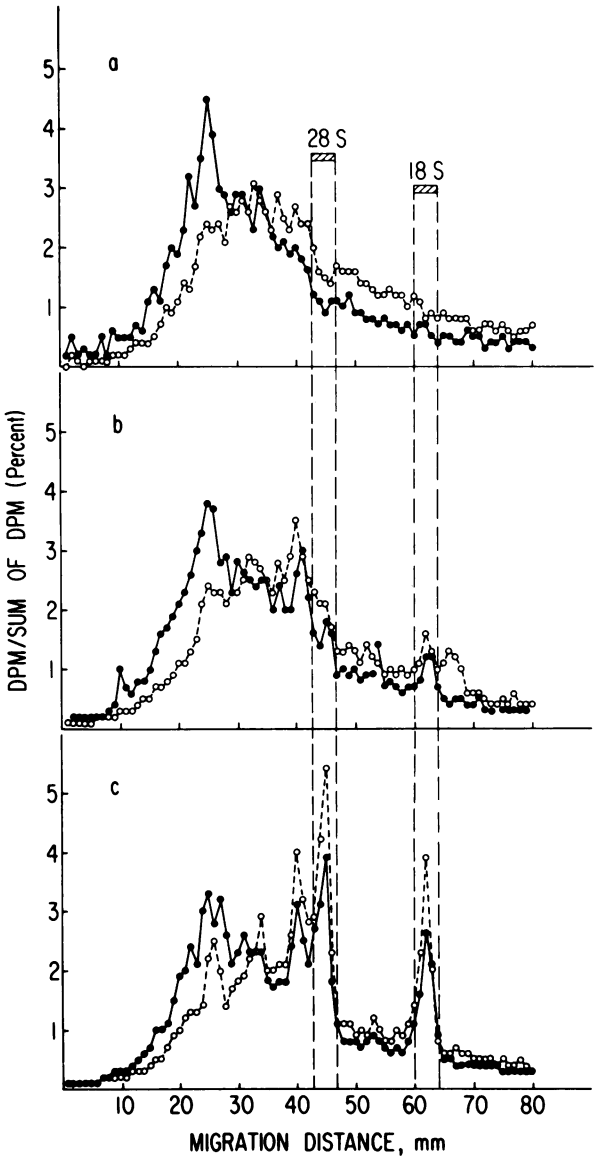

FIGURE 9. Electrophoretic profile of species of high molecular weight RNA synthesized *in vitro* in tracheal epithelium of normal and vitamin A-deficient hamsters. Distributions after labeling with [^{3}H]-uridine: (*a*) 30-min pulse; (*b*) 30-min pulse with additional incubation for 30 min; (*c*) 30-min pulse with additional incubation for 90 min; (○) RNA from tracheal epithelium vitamin A-deficient hamsters; (●) RNA from tracheal epithelium of vitamin A-normal hamsters. From Kaufman et al. (*88*) with permission of the authors and the American Association for the Advancement of Science.

processing or maturation of RNA, the effect of three inhibitors of specific steps in RNA synthesis were evaluated in organ culture. Toyocamycin, which inhibits the maturation of 18S and 28S ribosomal RNA species from their 45S precursor, actinomycin D and α-amanitin, inhibitors of RNA transcription and RNA polymerase II, respectively, were used to further characterize the effect of vitamin A deficiency. These compounds affected the RNA profiles in a manner consistent with their respective modes of action as described in other systems (*73*), therefore excluding a specific role for vitamin A in ribosomal RNA synthesis or processing. It was concluded that the effect of vitamin A deficiency was predominantly at the level of the heterogeneous nuclear RNA; these species of RNA are presumed to relate to messenger RNA. Since vitamin A deficiency is manifested as a change in the state of differentiation of this tissue, from a mucus producing to a squamous epithelium, it is plausible that an effect on messenger RNA could underlie or reflect the vast phenotypic changes seen in these cells.

Glycoprotein Biosynthesis

A major histologic change in respiratory epithelium of rodents deficient in vitamin A is a loss of secreting cells (*74*) whose secretory product is mucus, a glycoprotein mixture. Consequently, it was important to determine whether glycoprotein biosynthesis is altered in tracheas of vitamin A-deficient animals. Using vitamin A-deficient hamsters and rats, Bonanni et al. (*10,11*) showed that the amount of a glycopeptide containing fucose, mannose, hexosamines, galactose, and sialic acids synthesized by the rodent tracheas in cultures was dependent on vitamin A. In vitamin A-deficient rat tracheas the level of synthesis was a third of that in normal tracheas. More intensive studies of the mechanism of action of vitamin A in tissues such as liver (*75*), which allow more sophisticated analyses, indicate that vitamin A can act as a carrier in glycosyl transfer reactions in membranes. Conversion of vitamin A to retinyl phosphate apparently enables it to interact with GDP-hexoses and link with the sugar moiety, releasing GDP. The suger-charged retinyl phosphate can transfer sugar moieties to membrane-associated glycoproteins. This mechanism for the mode of action of vitamin A could explain the loss or reduction of glycoprotein species in vitamin A deficiency (*7,10,11,76,77*) as the result of a decreased capacity to manufacture mucus glycoproteins with specific sugar moieties. Further support for a role for vitamin A in glucoprotein synthesis came from work by Clark and Marchok (*76,77*). They reported that the type of mucins secreted by vitamin A-deficient rat tracheas in culture had an altered serine/glucosamine ratio.

Tracheal Organ Culture for Bioassay of Retinoids

Because of the interest in retinoids as possible cancer chemopreventive agents, it was necessary to develop a means for determining the potency and toxicity of retinoids in a relevant tissue. The ability of vitamin A to reverse morphologic alterations in vitamin A-deficient tracheal epithelium within days of administration was used by Sporn et al. (*35,36,38–40,78*) as an assay for retinoid potency. Tracheas excised from animals deprived of vitamin A and maintained in a serum-free, defined medium for 3 days were suitable for evaluating the effects of retinoids added to the medium. If an active retinoid such as retinoic acid were added to the medium at levels as low as 10^{-11}M, the squamous metaplasia and keratohyaline granules which indicated vitamin A deficiency diminished within 2 weeks, and normal morphology was restored. A quantitative estimate of efficacy was achieved by scoring a number of replicate cultures for the percent of cultures in which squamous metaplasia was reversed (Fig. 10). All-*trans*-retinoic acid was the most potent natural derivative of over 80 retinoids tested (*36*). Its 50% effective dose was 3×10^{-11}M (*36*). Tracheal epithelium in organ culture was approximately 10,000 times more sensitive to retinoic acid than were fibroblasts in cell culture (*79*). This indicates the need to evaluate the efficacy of such specific agents in the tissue in which its activity is relevant. Numerous retinoids have been synthesized and evaluated in order to identify compounds which are efficacious at low doses but are also free from toxicity. It appears as if the presence of the carboxylic acid, the alkene side chain, and the six-membered β-cyclohexenyl ring are all important determinants of the activity of retinoids; however, no simple

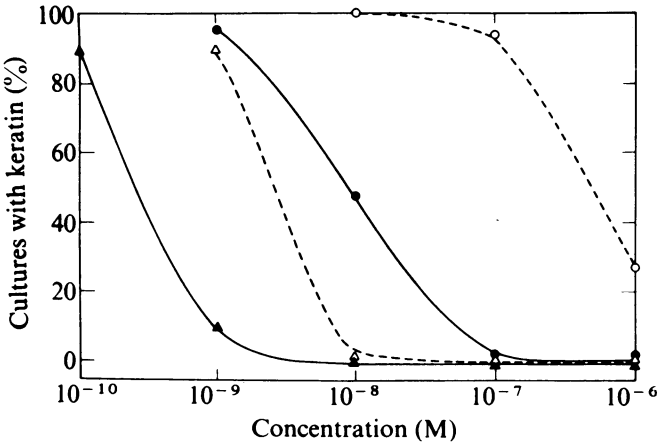

FIGURE 10. Bioassay for reversal of keratinization in tracheal epithelium in organ culture. Tracheas were incubated for 7 days in the presence of retinoids prior to scoring. Dose-response curves for: (▲), retinoic acid; (Δ), retinyl acetate; (●), *N*-acetyl retinylamine; (○), *N*-retinyl phthalimide. From Sporn et al. (*40*) with permission of the authors and MacMillan Journals, Ltd.

structure activity relationship is presently recognized. Single substitutions in any of these regions can result in a molecule with a markedly lowered biological activity. Conversely, there now exist a number of synthetic retinoids that are modified on both the ring and side chain, yet display a potency greater or equal to that of retinoic acid (*36*).

Metabolism of Retinoic Acid

The work of Sporn and collaborators (*34,35,37,80*) has elucidated another important aspect of the action of retinoic acid: that retinoic acid, like many xenobiotics and lipid-soluble compounds, can be metabolized in the target tissue. For retinoic acid, this biotransformation is linked to cytochrome P-450 containing mixed-function oxidase since the metabolism is sensitive to α-naphthoflavone (7,8-BF), is NADPH-dependent, is inhibited by carbon monoxide and it is inducible (*37*). It has previously been shown that retinoids act as competitive inhibitors of the mixed-function oxidase in lung microsomal incubations using BP as the substrate (*81*).

Like many substrates for the mixed-function oxidase, retinoids can act as inducers for their own metabolism by this enzyme complex. The magnitude of induction is dependent upon the particular retinoid, and the extent of induction varies from tissue to tissue. Interestingly, the classical cytochrome P-450 and P-448 inducers, phenobarbital and MCA, are ineffective as inducers of retinoic acid metabolism.

Roberts et al. (*37*) assayed for retinoid-induced metabolism of retinoic acid and derivatives *in vivo* and *in vitro* using hamster kidney, liver, trachea, testis and intestine. Vitamin A-deficient hamsters were given retinoids orally for induction for 3 days, after which [^{3}H]retinoic acid was injected intrajugularly and tissue excised after 4 hr. The metabolism of retinoic acid was found to be inducible *in vivo* in liver, kidney, and intestine. Both the testis and trachea had a high metabolic capacity for retinoic acid; however, the activity was independent of induction (i.e., was not inducible). Vitamin A-normal animals were much more metabolically competent at retinoic acid metabolism than the deficient animals, indicating that some degree of induction is present in the normal nutritional state.

The metabolism of retinoic acid has been characterized in detail in hamster tracheal organ cultures by Frolik et al. (*34,35*). Two major metabolites were detected after separation on reverse-phase HPLC. Derivativization and subsequent mass spectral analysis indicate that. one metabolite is 4-hydroxyretinoic acid and the other is 4-oxoretinoic acid. When assayed in tracheal organ culture, both of these compounds exhibit little biological activity, and are on the order of 3% as active in reversing keratinization as is retinoic acid. Therefore, these metabolites were not considered "active intermediates" responsible for the activity of retinoids, and are likely to be products of inactivating pathways for retinoids (*34,35*).

Conclusion

The preceding discussion has focused on two areas of progress in studies of the biochemistry of tracheobronchial epithelium. As noted earlier, these topics were the subjects of investigations because biological studies of carcinogenesis in the rodent respiratory tract had revealed that carcinogen metabolism and effects of retinoids were important contributing factors. Within the framework provided by the biological observation of carcinogenesis studies, biochemical studies in both areas could proceed in an effort to relate biochemical mechanism to biological observation. Studies of carcinogen metabolism appear to have provided a mechanistic explanation for the vast difference between two species in susceptibility to tracheobronchial carcinogenesis by BP (*13,14*). They also may be providing an explanation for the specific risk of certain individuals to lung cancer. Furthermore, other studies in tracheobronchial epithelium have provided initial or important contributions to knowledge regarding the metabolism of BP-quinones (*21*), the mechanism of action of vitamin A (*82,83*), and the alterations in RNA metabolism related to reprogramming of cells for an altered state of differentiation. Several of these lines of investigation have proceeded with technical methods that were highly sophisticated and comparable to those used with other cells or tissues where quantities of material were far less of a problem.

In addition to providing a record of progress, these studies also illustrate a field of opportunity. Although studies with tracheobronchial epithelium still may be difficult because of the time and expense of obtaining the tissue, once the tissue is available, a wide range of biochemical investigations can be performed. Where a specific problem must be evaluated directly in this tissue, the methods exist for many types of studies and the opportunity exists for further development. Initial efforts were made based upon observations in respiratory carcinogenesis studies but the approach taken to study the biochemical features of carcinogenesis models can be applied to investigations of the pathogenesis of other diseases in this tissue.

With the development of techniques for the establishment of primary cell cultures and lines of bronchial and tracheal epithelial cells (*29–32,84–87*), the opportunities for the study of the biochemistry of the tracheobronchial epithelium may by greatly expanded. In addition to the reduction in cost resulting from the use of cultured cells rather than large numbers of animals, the obtainment of cells is much easier and the cell populations are far more homogeneous than those collected from animals. Furthermore, the availability of cell lines at various stages in the progression from normal to malignant may provide exciting opportunities for study

of the critical biochemical changes in premalignant and malignant tracheobronchial epithelial cells.

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